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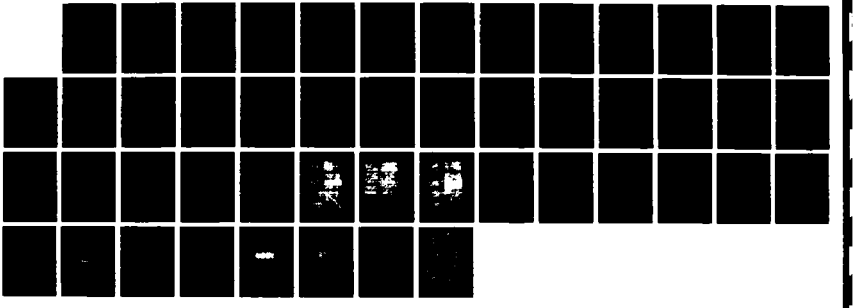

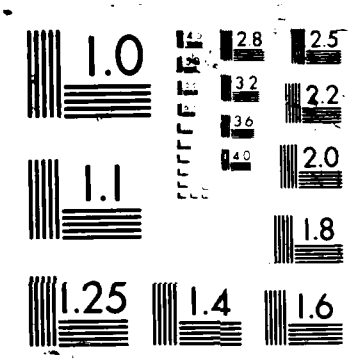
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DEVELOPMENT AND APPLICATION OF NUCLEIC ACID HYBRIDIZATION TECHNIQUES TO ARBOVIRUS SURVEILLANCE AND DIAGNOSIS

FINAL REPORT

AUGUST 14, 1987

MARCH 1, 1985 - AUGUST 14, 1987

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Nucleic acid hybridization techniques have been developed for detection of LaCrosse and dengue virus RNA in cells and in mosquitoes. RNA transcript or cDNA probes of the respective viruses were prepared and labeled with either biotin or a radioisotope. Optimal conditions for cell fixation, permeabilization, retention of analyte, hybridization, and detection of hybrids for in situ hybridization were determined for detection of LaCrosse virus analyte in cells. La Crosse RNA was first detected 4 hours post infection of cells, and as little as 700 copies of analyte could be readily detected. Both type-specific (a cDNA of the M RNA segment of a bunyavirus) and group-specific (a cDNA of the S RNA) have been developed. Radiolabeled probes have been emphasized for dengue virus detection. Studies demonstrated inefficient binding of RNA analyte to the substrate. Novel strategies to overcome this problem, UV crosslinking of analyte to substrate and sandwich hybridization, have been developed. Using the former technique, RNA from on infected mosquito in a pool of 25 negativ could be readily detected using RNA transcript probes. This represented approximately

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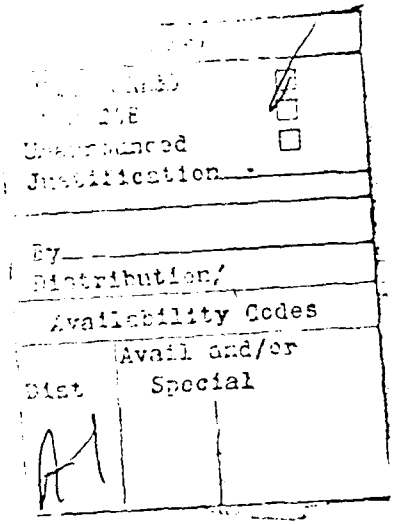
19. Abstract, Continued.

525 infectious virus particles or 0.14 pg of virion RNA. Sandwich hybridization techniques have been developed for detection of dengue virus RNA. Capture and detector probe constructs have been prepared in M13 and in vitro transcription plasmids, respectively. Nanogram levels of dengue RNA have been readily detected using the technique.



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Summary

A molecular hybridization technique was developed to detect LaCrosse (LAC) virus in BHK-21 cells. A complementary DNA (cDNA) to the S RNA and a portion of the snowshoe hare (SSH) M RNA were cloned into the PvuII and the PstI sites of the plasmid pBR322. Specificity of probes was determined by using ^{35}S labeled DNA in an in situ hybridization procedure. Probes were hybridized to cells infected with California group viruses. The S RNA cDNA probe hybridized to all of the viruses tested. Thus it is a group-specific probe. The cDNA of SSH virus M hybridized to SSH infected cells, but not to LAC infected cells. Thus, the M RNA cDNA is a type-specific probe. RNA transcript probes have also been developed and used to detect LAC RNA extracted from infected cells. The sensitivity of the RNA probe has been determined.

For nonisotopic probes the recombinant S RNA plasmid was labeled by enzymatic incorporation of biotinylated dUTP and used as a probe. Optimal hybridization and detection parameters were determined, resulting in the following protocol: cells grown to confluency on subbed slides and infected with LAC virus were fixed in paraformaldehyde, permeabilized with pronase (Calbiochem, 50 ug/ml) and hybridized with the labeled, denatured probe overnight at 37C. After hybridization, cells were treated sequentially with the following reagents to detect hybridized probe: 1) goat anti-biotin antibody, 2) biotinylated rabbit anti-goat antibody, 3) avidin-biotinylated horseradish peroxidase, and 4) substrate (0.2mg/ml DAB - 0.06% H_2O_2).

Virus antigen was first detected by direct immunofluorescence in a few cells at 2 hours post infection; by 6 hours, most cells were expressing substantial antigen. The presence of specific hybrids was first detected in the perinuclear region of cells at 4 hours PI. By 6 hours, hybridization signal was detected in most cells. By 12 hours signal was detected in more distal portions of the cell, and by 24 hours intense hybridization signal was detected throughout the cytoplasm.

For dengue virus hybridization, the three cDNA clones pVV1 (1.4 kb dengue insert), pVV9 (1.65 kb insert), and pVV17 (1.95kb insert) to nonstructural regions of dengue-2 virus genome sequences were obtained from Dr. Radha Padmanabhan at the University of Kansas and were grown and amplified by standard procedures. Plasmids were extracted, quantified, and analyzed for the correct constructs by gel electrophoresis. The recombinant plasmids were biotin-labeled by nick translation in which dTTP was replaced by bio-11-dUTP.

The three biotinylated probes were used in preliminary hybridization studies to determine sensitivity and specificity of each. Crude cell culture supernatant, cytoplasmic RNA preparations, and extracted viral RNA were blotted onto nitrocellulose and hybridized with each of the probes. Hybridized dengue cDNA was detected by immunobiochemical techniques. The largest probe, pVV17, proved to be the most sensitive, detecting picogram levels of extracted dengue-2 viral RNA with little reactivity with cellular RNA. The other probes, pVV1, and pVV9, appeared to react to a greater extent with cellular preparations. However, when the three inserts were isolated from the plasmids, nick translated using ^{32}P-dCTP, and hybridized

to dengue virus RNAs blotted onto nitrocellulose, no cross reactions with cell preparations were noted. The pVV1 derived probe was the most sensitive and detected 32 pg of dengue-2 RNA, 160 pg of dengue-3, and 20 ng of dengue-4. Dengue-2 and dengue-3 RNAs were extracted from Aedes albopictus cells. The pVV9 and pVV17 probes were more specific for dengue-2 RNA, but some cross reaction with other dengue serotypes was noted.

Sandwich hybridization procedures were developed and tested for detection of dengue virus RNA in cell or virus extracts. All or part of the pVV-17 1.95 kb cDNA was subcloned into in vitro transcription plasmids or single stranded (SS) DNA producing phage to construct 8 highly specific nucleic acid probes. Hybridization probes using cDNA from pVV-1 and pVV-9 were also constructed. ^{32}P labeled probes were used to lay the groundwork for hybridizations employing nonradioactive probes. The radiolabeled probes detected less than 100 pg of genomic dengue RNA or approximately 1×10^6 genome equivalents of dengue nucleic acid. A novel sandwich hybridization technique was developed to detect dengue RNA sequences in unprocessed clinical specimens. This hybridization technique utilized SS RNA and SS DNA probes to function as detector and capture molecules, respectively, in the sandwich assay. The results of the first trials using sandwich hybridization were most encouraging; nanogram levels of viral RNA were readily detected. Studies were begun to determine the sensitivity and specificity of the technique as well as its applicability to detection of viral nucleic acid in crude specimens.

Foreword

In conducting this research, the investigators have abided by the National Institutes of Health Guidelines for Research Involving Recombinant DNA molecules (May, 1986).

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BODY OF THE REPORT

I. Statement of the problem

Studies were proposed to develop, to evaluate, and to apply nucleic acid hybridization probe methodology to the surveillance and diagnosis of arboviruses in vectors or vector populations. Special attention was to be devoted to development of methods that can be applied in field circumstances. In particular, biotin-labeled hybridization probes (BLHPs) which can be detected by enzyme immunoassay techniques, were to be emphasized. Sensitivity and specificity of BLHP methods were to be compared and contrasted with conventional radioisotopic hybridization probe procedures as well as virus isolation and antigen detection techniques.

II. Background

Surveillance programs permit identification of geographical areas of high risk for arbovirus infections. Surveillance programs for arboviruses usually entail monitoring of either infections in humans and/or other vertebrates and monitoring of vector populations and infection rates (1). Estimates of vector population densities and infection rates are particularly useful for predicting the risk of human and animal disease. Since such information can be gathered before the incidence of human disease, health practitioners can successfully intervene to prevent infections.

The conventional method for arbovirus surveillance involves isolation of virus from mosquitoes or mosquito pools by amplification in a bioassay such as suckling mice, cell cultures, or embryonated eggs (1). Virus isolates must then be serologically identified. These conventional techniques are labor intensive, slow, and may require elaborate laboratory tissue culture and containment facilities. Frequently samples must be collected and sent to distant central laboratories for processing. Results typically are not available in time to permit field practitioners to institute effective control or therapeutic measures. Further, samples must be protected from environmental conditions which could destroy virus infectivity. Thus elaborate cold-chains or other protective measures are necessary to ensure virus viability for amplification.

Second generation diagnostic tests circumvented several of these problems. In these tests, diagnosis is effected by detection and simultaneous identification of virus specific antigens. Enzyme immunoassays (EIAs) are examples of second generation diagnostic procedures which preclude many difficulties associated with conventional virus isolation and subsequent identification techniques (1, 2). However, certain technical and methodological difficulties are inherent in EIA techniques. Many of these can be precluded by the use of a third generation diagnostic test, nucleic acid (NA) hybridization. Since direct detection of NA is the method of diagnosis, concerns with antigen-antibody equivalence, antigen clearance or degradation, latent infections or other infections without substantial antigen production, and specificity of immunoglobulins are all eliminated. Nonetheless, the sensitivity and specificity of EIA may be retained. A

drawback to the widespread use of hybridization in diagnosis has been the need for radioisotopic labeling of probes. However, development of BIHPs by Ward and co-workers (3, 4) provides a technique that retains the exquisite sensitivity and specificity of nucleic acid hybridization without drawbacks associated with safety, shelf-life, and disposal of radioisotopes. After hybridization, the biotin reporter molecule incorporated into a probe can be detected immunologically by immunofluorescence (IF) or enzyme immunoassay (EIA). Biotinylated NA hybridization techniques have gained wide acceptance for detection of both integrated and extrachromosomal virus specified NA sequences (3, 5-7). However these techniques had not been applied to the detection of arboviruses in vectors.

The rationale for this proposal was that NA hybridization techniques should be applicable to detection of arbovirus genomes or specified NA sequences in vectors. Further, NA hybridization should provide an extremely sensitive and specific mechanism for detection of infected arthropods. With the substitution of biotin-labeled NA probes for radioisotopic probes, the technique should be adaptable to field situations and should permit rapid diagnostic capability. Further, immobilization of virus NA on nitrocellulose should permit storage and transport of diagnostic samples, thereby precluding problems of preservation of virus antigens or infectious virus.

III. Methods and Results

A. LaCrosse (LAC) virus: Development of techniques for detection of virus nucleic acid (NA) species:

In the LAC virus system, progress was made in development of type and group specific hybridization systems. In addition, studies were begun to develop SS RNA probes (SP6 transcripts) for detection of LAC nucleic acids (9).

Initially radioactive probes were used to more accurately determine hybridization parameters in order to develop a reliable test and to permit quantitation of levels of RNA in cells. Subsequently, biotinylated probes were developed for both in situ hybridization and detection of LAC RNA in blots from cells and mosquito suspensions. In both types of specimens the cDNA of the S RNA of LAC virus cloned into the PvuII site of the plasmid pBR322 was used as a probe (8). The probe was labeled with ^{35}S nucleotides or biotinylated-dUTP by nick translation.

1. Hybridization - specificity of ^{35}S labeled S RNA cDNA probe.

Studies were conducted to determine the specificity of the LAC S RNA probe. The construct was labeled with ^{35}S by nick translation. Cells were infected with the following California group viruses: LAC, SSH, California encephalitis virus (CEV), tahyna (TAH), Jamestown Canyon (JC), and trivittatus (TVT). After 24 hours of incubation, cells were fixed in 4% paraformaldehyde and stored in 70% ETOH. For hybridization, slides were removed from ethanol, rehydrated in PBS, and permeabilized by incubating for 10 min at room temperature (RT) in 50 ug/ml of nuclease free protease (Pronase, Calbiochem). Viral RNA was denatured by incubation of the slides in 2xSSC-50% formamide at 65C for 10 min., then cells were prehybridized for one hour at room temperature in hybridization buffer without probe. This

buffer consisted of 45% formamide, 2xSSC, 10% dextran sulfate, 200 ug/ml herring sperm DNA, 10mM Vanadyl Ribonucleoside Complex (VRC, Bethesda Research Laboratories), 1X Denhardts (0.02% w/v each Ficoll, PVP, and BSA), and 2ug/ml yeast tRNA. Slides were hybridized in the same buffer plus 1ug/ml ^{35}S labeled probe and 100mM dithiothreitol (DTT). DTT was necessary to prevent nonspecific adherence of the ^{35}S to the cells or the slide. The specific activity of the probe was 1-2 x 10^6 cpm/ug. Hybridization was at 37C for 16 hours. After hybridization, slides were washed in 2xSSC-45% formamide twice for 10 min each at 37C, once in 2xSSC at 37C for 10 min, and once in 2xSSC for 10 min at RT. Slides were dehydrated through an alcohol series, and hybridized probe was detected by autoradiography. For autoradiography, Kodak NTB-2 nuclear track emulsion was melted at 45C, slides dipped in the liquid emulsion, and allowed to dry at RT for 1 hour. Exposure was at 4C for 3 days. Silver grains were developed by placing the slides in Kodak Dektol developer diluted 1:1 with water, for 2 min; stopping in water for 10 seconds, fixing in Kodak fixer for 5 min, and rinsing in water for 5 min. All of these reagents were at 15C. Slides were air dried overnight; then counterstained with Mayers hematoxylin. Hybridized probe was seen by the presence of black grains over the cells.

The probe strongly hybridized to LAC infected cells but not uninfected cells (Figure 1). The probe also hybridized to all of the tested California group viruses (Figure 2), including TVT virus which is only distantly related serologically. This was not unexpected because the S RNA is considered to be the most conserved portion of the bunyavirus genome.

2. Hybridization - specificity of ^{35}S labeled SSH M RNA cDNA.

Studies were subsequently conducted to determine the specificity of a cDNA of a portion of the M RNA segment. The rationale was that since the M RNA codes for the envelope glycoproteins it would be more likely to be type specific. The probe (SSH M60-78) was a cDNA copy of a 2161 bp portion of the SSH M RNA segment cloned into the PstI site of pBR322 (14). The construct was labeled with ^{35}S by nick translation. BHK-21 cells were infected with LAC or SSH viruses. Cells were harvested, processed, and hybridized as related above. Autoradiography protocols were also identical.

Interestingly, hybridization signal was only detected in cells infected with SSH virus; cells infected with LAC virus were negative (Figure 3). Thus the middle RNA probe is type specific.

3. Blot Hybridization - LAC RNA probes.

The 569-bp Pst I fragment of the cDNA of LAC virus small RNA segment was cloned into the Pst I site of the plasmid vector pSP65 (Promega Biotec). This plasmid contains the bacteriophage SP6 promoter (9), a multiple cloning region, and an ampicillin resistance gene. E. coli HB101 were transformed with the recombinant plasmid and selected for ampicillin resistance.

Plasmid was purified from large-scale cultures of transformed E. coli by a standard alkaline lysis procedure, and linearized with the restriction enzyme Hind III. Linearized SP65-LAC plasmid was used as template for the in vitro transcription of RNA by SP6 RNA polymerase. Labeled RNA was prepared by incorporating either ^{32}P or ^{35}S UTP. DNA template was removed by treatment with DNase, and labeled RNA recovered by ETOH precipitation. This labeled RNA was used to detect LAC virus RNA which was blotted onto

nitrocellulose.

RNA was purified from LAC virus infected cells by phenol-chloroform extraction. RNA was blotted onto nitrocellulose (10) in dilutions and fixed at 80C in vacuo for 2 hours. Blots were prehybridized in a solution consisting of 50% formamide, 5xSSC, 25 mM $NaPO_4$, 5X Denhardts solution, 250 ug/ml sonicated salmon sperm DNA, 0.1% SDS, and 200 ug/ml yeast tRNA, for 4 hours at 42C. Blots were hybridized in the same solution plus 5 x 10^6 CPM ^{32}P labeled RNA or 3 x 10^6 CPM ^{35}S labeled RNA, overnight at 42 C. After hybridization, blots were washed extensively, dried, and exposed to X-ray film. ^{32}P blots were exposed for 3 days, and ^{35}S blots were exposed for 5 days. Both the ^{32}P and ^{35}S labeled probes were equivalent in sensitivity (Figures 4 and 5).

4. In situ hybridization using biotinylated probes.

The complete protocol used for in situ hybridization is presented in the appended manuscript. In brief, cells were propagated on subbed slides, infected with LaCrosse virus (MOI=10), fixed in paraformaldehyde, treated with pronase and then hybridized with the probe. After hybridization, slides were successively treated with goat anti-biotin Ab, biotinylated rabbit anti-goat Ab, ABC complex, and DAB. In infected cells, the converted substrate was seen as a brown precipitate in the cytoplasm of the cells.

The technique was used to determine the time and cellular localization of synthesis of the S RNA species of LAC virus in BHK-21 cells. Results were contrasted with immunofluorescent detection of virus antigen in the cells (Figures 6, 7, and 8). Hybridized probe was first detected 4 hours post infection; the signal was not intense and was restricted to distinct perinuclear areas. By 12 hours, strong signal was detected in perinuclear spaces and signal was detectable in more distal regions of the cytoplasm. By 24 hours, intense signal was detected throughout the cytoplasm. A few cells contained detectable virus antigen by 2 hours post infection. Most cells were expressing significant amounts of antigen by 4-6 hours.

Studies were also conducted to simplify and enhance the sensitivity of the in situ hybridization procedure using biotin probes. Plasmid DNA was labeled with biotin-11-dUTP by nick translation. Several systems for the detection of biotinylated DNA hybrids were tested. Biotin-labeled probes were detected directly with avidin-biotin complex (ABC), or with indirect immunocytochemistry. For the direct system, two types of ABC complexes were used: ABC-AP (alkaline-phosphatase enzyme), and ABC-HRP (horseradish peroxidase enzyme). Both direct systems resulted in signal development in the virus infected cells with little or no background signal. With either enzyme, the indirect system resulted in more intense signal. Several types of substrates are available for use with AP. Substrate I, which results in a black precipitate upon conversion by the enzyme, was used in the detection of virus infected and uninfected cells. Cells were hybridized with a biotinylated probe, and hybrids were detected directly with ABC-AP (Figure 9).

The comparative sensitivity of biotin probes detected with the indirect immunocytochemistry (ABC-HRP), ^{35}S-labeled probes, and antigen detection with monoclonal antibodies directed against the nucleocapsid protein, G1 glycoprotein, and polyclonal antibody were determined in a growth curve of LAC virus in BHK-21 cells. Cells were infected with virus, and harvested

every 2 hours post infection for 12 hours. At 0 hours PI, there was no antigen detectable, but input virus RNA was detectable with the ^{35}S labeled probe (Table 1). At 2 hours PI, a small amount of antigen was detectable but RNA was not detectable. RNA became detectable with the ^{35}S labeled probe at 4 hours PI, but not until 6 hours with the biotin labeled probe. The radiolabeled probe was slightly more sensitive than the biotin labeled probe.

5. Dot/blot hybridization using biotinylated probes

Dot/blot hybridization protocols were developed using LAC viral RNA extracted from supernatants of infected cell cultures. After PEG precipitation and centrifugation, viral pellets were resuspended in STE buffer containing SDS. After phenol:chloroform:isoamyl alcohol and subsequently chloroform:isoamyl alcohol extraction, the RNA was recovered by ethanol precipitation. The RNA was pelleted, dried *in vacuo*, and resuspended in 10xSSC. The concentration of RNA was determined by UV absorption. To prepare blots, dilutions of RNA were made in 15xSSC-7.4% formaldehyde, incubated at 65C for 15 min, then blotted onto nitrocellulose (10) using a slot blot apparatus. The nitrocellulose sheets were then baked *in vacuo* for 2 hours at 80C. Blots were pretreated to prevent nonspecific binding of probe, and then hybridized in 50% formamide, 5xSSC, 25 mM $NaPO_4$, 1xDenhardts solution, 250 ug/ml carrier DNA, 10% dextran sulfate, and 0.1ug/ml labeled probe for 16 hours at 42C. After hybridization, sheets were washed, blocked, and the detection system applied. Detection steps included steptavidin , biotinylated poly-alkaline phosphatase, and NBT-BCIP substrate. These were added for 10 minutes, 10 minutes, and 3-4 hours, respectively, with intermittent washes. Development of color was stopped by washing the blots in TRIS-EDTA.

Mosquito suspensions were similarly treated with the omission of PEG precipitation and centrifugation. Mosquito processing began with the phenol:chloroform extraction step.

When LAC virus NA was blotted onto nitrocellulose, 100 ng of RNA was detected readily (Figure 10). However, this technique undoubtedly greatly underestimated the sensitivity of the probe. For example, the efficiency of LAC RNA binding to the nitrocellulose was not known. In this procedure, probe only detects S RNA species; L and M species were not detected. In addition, the blot represented only a crude cell culture supernatant extract; thus the estimated amount of RNA probably included cellular RNA. Nonetheless the results were encouraging.

As expected, the LAC S RNA cDNA probe did not differentiate between California group viruses (Data not shown). S RNA species from SSH, TAH, TVT, JC, and CE viruses cross-hybridize. The lack of specificity with the S RNA species was not unexpected, because it is the most conserved portion of the genome.

The probe was also capable of detecting S RNA species in infected mosquitoes (Figure 11). Two pools each of 10 infected or noninfected mosquitoes were processed as described. After extraction, RNA from infected and noninfected mosquito suspensions was blotted onto nitrocellulose and processed as noted previously. As can be seen in Figure 11, substantial signal resulted from the infected pools. Some signal was detected in the

negative pools, but the intensity of the signal was dramatically higher in the positive pools.

B. Dengue virus: development of techniques to detect NA species:

For detection of dengue virus by hybridization, most effort was directed to detection of virus nucleic acids in suspension. Both biotinylated and radiolabeled cDNA and RNA probes were developed and used to detect virus in blot hybridization protocols.

1. Dengue blot hybridization: biotinylated probes

Blot hybridization techniques were developed for the detection of virus RNA species in cell extracts. Three plasmids containing cDNA sequences of the virus genome cloned into the Pst I site of pUC13 were obtained from Dr. R. Padmanabhan of the University of Kansas. The three recombinant plasmids, pVV1 (1.4 kb insert), pVV9 (1.65 kb insert), and pVV17 (1.95 kb insert) were biotin labeled by nick translation with biotin-11-dUTP. The probes were compared for sensitivity and specificity in hybridization studies using crude cell culture supernatants, cytoplasmic RNA preparations, and extracted viral RNA. The probes were hybridized to the respective samples blotted onto nitrocellulose (9). The largest probe, pVV17, has proved thus far to be the most sensitive, detecting picogram levels of extracted dengue-2 viral RNA with little cross reactivity. For unknown reasons, the other two probes exhibited greater cross reactivity with cellular extracts.

2. Dengue blot hybridization - radiolabeled cDNA probes

To investigate further the specificity of the probes, the three cDNA inserts were isolated from the plasmids and nick translated using ^{32}P-dCTP. These probes were hybridized to RNAs extracted from purified virus and infected C6/36 mosquito cell lysates. RNAs extracted from uninfected cell lysates served as one negative control and blotting of solutions containing no RNA served as another. RNAs were blotted onto nitrocellulose using a dot blot manifold apparatus in high salt conditions (10xSSC). Following denaturation in the presence of formaldehyde (7.4%) at 65C for 15 minutes, blots were baked at 80C for 2 hours. Hybridization was at 42C for 48 hours. For autoradiography, blots were exposed to x-ray film overnight at -70C. Longer exposures may well increase the sensitivities noted below.

The hybridization results corroborate that all 3 clones contained specific inserts that strongly hybridized to dengue RNA and to the RNA of other serotypes to varying degrees (Figure 12). The pVV1 derived probe detected 32 pg of dengue-2 RNA, 160 pg of dengue-3 viral RNA, and 20 ng of dengue-4 RNA. The pVV1 probe also detected dengue RNA sequences extracted from dengue 2 and 3 infected C6/36 cells. The pVV9 probe was dengue-2 specific and detected 4 ng of RNA. The pVV17 probe was primarily dengue-2 specific, detecting 800 pg of viral RNA. Some hybridization was noted with RNAs extracted from dengue 1 and 3 viruses. The pUC-13-1 probe (non-recombinant plasmid control) only hybridized to pUC-8 and not to any RNAs, indicating that the pUC-13-1 plasmid vector is not a source of nonspecificity. None of the probes hybridized to the negative controls. Thus, non-specific binding of plasmid probes can not be attributed to either the vector DNA or the cDNA inserts and may instead be due to the system used for detection of hybrids. Also, the cDNA sequences vary in both specificity

and sensitivity for detection of dengue-2 and the other dengue virus serotypes.

3. Inefficiency of dengue RNA immobilization on nitrocellulose:

To determine the efficiency of RNA attachment to nitrocellulose, ^{32}P labeled dengue RNA was transcribed from the KO65-1200 DNA template (see below). One microliter of the labeled transcript was mixed with 1 ug of denatured salmon sperm DNA and applied to nitrocellulose in high salt (10xSSC) using a dot/blot apparatus (10). Counts per minute were determined before and after application of samples to nitrocellulose. Counts were also assessed after a mock hybridization with subsequent washes to determine RNA finally retained on the filter. Results are summarized in Table 3. Briefly, For all samples, more than 80% of the RNA eluted from the nitrocellulose.

4. UV immobilization of dengue analyte on nitrocellulose

Studies have been conducted to determine if direct UV crosslinking of analyte to substrate could preclude difficulties with elution of dengue RNA during hybridization. Briefly the technique allows the covalent binding of NA to a nylon filter by ultraviolet irradiation. The thymine or uridine residues of the NA are covalently linked to the primary amine groups of the nylon filter. Linkage is accomplished by exposure of a wet filter containing the NA 15cm above a UV source (300 nm transilluminator) for 3-4min. Picogram quantities of NA have been detected. This technique does not require extensive drying and baking of the filter to fix NA to the filter.

The direct detection of dengue RNA in infected mosquitoes has been tried by extracting RNA from pools of mosquitoes with various ratios of infected to noninfected vectors. Mosquitoes were intrathoracically inoculated with dengue-2 and after the extrinsic incubation (EI) head squashes were assayed with fluorescent antibody to assess infection. Mosquito abdomens identified as DEN positive by the above assay were stored at -70^0C until needed. Each pool contained 25 mosquito abdomens which were triturated in a lysis buffer containing urea and SDS. Lysed mosquitoes were extracted twice with equal volumes of phenol followed by two chloroform extractions and an ethanol precipitation. Precipitated RNA was resuspended in 100ul of water and frozen at -70^0C. RNAs were denatured in 7% formaldehyde and 6X SSC for 15 min at 65^0C. Ten ul of each sample was applied to a nylon filter on a slot blot apparatus. NA was cross-linked by UV irradiation as previously described. Hybridization occurred overnight at 42^0C in 50% formamide, 6X SSC, and 0.1% SDS using a ^{32}P-CTP labeled RNA probe (KO64-750). The RNA probe consists of a 750bp cDNA fragment from the NS-5 gene region of DEN-2 RNA ligated into pSP64 transcription plasmid. Labeling procedures have been previously described. Washes involved four 5min washes in 2XSSC/0.1%SDS at room temperature and two 15min washes in 0.1XSSC/0.1%SDS at 50^0C. The binding of nonspecific RNA probe was reduced by incubating the blot in 10ug/ml of RNase A. After a 7 day exposure 1 infected mosquito in 25 could be easily detected (figure 13). These results have been compared with virus titrations of initial inoculum and isolation and titration of virus following EIP. Sensitivities of direct detection and sandwich hybridization will be compared as well as efficiencies of radiolabeled probe and biotinylated probes.

5. Dengue virus isolation from mosquito pools

After incubation for 10 days, mosquitoes infected with dengue-2 virus were used to construct pools A-E (Table 4). Mosquito pools were triturated in 10 ml of a trituration diluent (L-15 medium, 500 U penicillin, 50 ug/ml streptomycin, 5 ug/ml fungizone). Serial 10-fold dilutions of each pool were combined with C6/36 cells, plated in quadruplicate chambered slides, and fixed in cold acetone and examined for virus antigen using a direct immunofluoresence technique. Titers of pools ranged from 5.25 $\log_{10}$ TCID50/ml (pool A) to 2.75 $\log_{10}$ TCID50/ml (pool D), representing minimally from 44 pg to 0.14 pg of genomic RNA. therfore as little as 525 infectious virus particles or 0.14 pg of virion RNA was detected by cross-linking RNA from pools of infected mosquitoes and hybridizing with strand-specific RNA probes. Row F (figure 13) represents 1 ng to 10 pg of purified viral RNA and suggests a sensitivity of detection of 10 pg of purified viral RNA. Thus the hybridization technique probably detects large quantities of intracellular dengue-2 viral RNA and perhaps RNA of defective viral particles.

6. Dengue sandwich hybridization

Sandwich hybridization methodologies have been reported to be sensitive and specific techniques for detecting nucleic acids (11,12). One important advantage of sandwich hybridization over conventional dot/blot hybridization schemes is that there is apparently no need to extract the target NA species. If true, such a technique would greatly facilitate detection of arbovirus NA species in mosquito pools. Accordingly, considerable effort was expended in developing a sandwich hybridization technique for dengue viruses. To accomplish this goal, dengue-2 specific single stranded (SS) RNA probes and SS DNA probes were generated by subcloning the 1.95Kb cDNA insert from pVV-17 obtained from Dr. R. Padmanabhan. The probes were made by subcloning all or part of the cDNA into an appropriate vector. Many of the constructs took advantage of a unique EcoRI restriction endonuclease site within the 1.95 Kb cDNA to produce fragments containing dengue-2 cDNAs of approximately 1200bp and 750 bp. Clone K064-750 was produced by subcloning the 750 bp fragment into the PstI and EcoRI sites in pSP64. Clone K065-1200 was constructed by subcloning the 1200 bp cDNA into identical restriction sites in pSP65. The two in vitro transcription plasmids, pSP64 and pSP65, differ only in orientation of their unique cloning sites with repect to the transcription promoter derived from the SP6 bacteriophage. This ensures that each cDNA is ligated into the correct orientation to produce anti-sense RNA complementary to the positive (+) sense genomic RNA of dengue virus. Anti-sense RNA is made by transcribing the DNA template in the presence of a specific SP6-phage encoded RNA polymerase. Clones K065-1950A and K065-1950B were constructed by subcloning the entire 1.95 Kb cDNA into the PstI site of pSP65. The two clones represent opposite orientations of the insert. K065-1950A DNA can be used to transcribe antisense RNA probes and K065-1950B DNA is used to transcribe positive (+) sense RNA which can be used to simulate viral RNA in optimizing hybridization conditions.

The M13 recombinant phages were produced by isolating double stranded DNA replicative form (RF) from infected E. coli (13). Clone K01318-750 and K01319-750 were constructed by subcloning the 750 bp cDNA into the PstI sites of the RF DNA. Clone K01318-750 and K01319-750 were derived from M-13 vectors M13mp18 and M13mp19 respectively. The two vectors differed only in the orientation of their unique cloning sites. Clone K01318-1200 and K01319-1200 were constructed similarly by subcloning the 1200 bp cDNA into

each M-13 RF. Phage produced by transforming E. coli with the two constructs, KO1319-750 and KO1318-1200, contain SS DNA having dengue specific sequences complementary to viral RNA. Figure 14a shows the original plasmid pVV constructions, and Figure 14b shows the relative locations of homologous cDNAs on the yellow fever virus genome. The in vitro transcription plasmids and M13 replicative form (RF) constructs which have been developed to date are shown in Figures 15 and 16. Similar constructs are being prepared using the pVV-1 and pVV-9 plasmid inserts.

Studies were begun to evaluate the efficacy of these probes for the detection of dengue-2 RNA sequences using sandwich hybridization. Briefly, the technique utilizes a nitrocellulose-bound M13 derived SS DNA catcher probe containing the dengue-2 sequences which hybridize to dengue-2 RNA from the nonstructural genomic region. Simultaneously, an SP6 derived SS RNA detector probe hybridizes to the genomic RNA at sequences adjacent to those recognized by the catcher probe. A schematic diagram of the technique is shown in Figure 17. Sandwich hybridization has several advantages in that it does not require the binding of RNA to nitrocellulose, which appears to be an inefficient process even in the presence of high salt. A second advantage is that relatively crude extracts of samples can be used during hybridization.

Preliminary tests using purified genomic dengue-2 RNA and dengue RNA of (+) polarity produced in vitro were encouraging. Figures 18 and 19 are autoradiographs of sandwich hybridizations in which 100 ng of viral RNA or transcribed RNA were detected. The amount of immobilized catcher probe necessary to anneal dengue viral RNA and form a sandwich hybridization complex were determined. Multiple trials, using serially diluted (1:2) single-stranded catcher probe (M13 recombinant phage KO1319-750) demonstrated that 0.2 to 0.5 ug of catcher was required for the best signal. Signal strength was assessed from autoradiographs both visually and by densitometry. Catcher probe was bound to a modified nylon membrane (Nytran) by UV-crosslinking. The advantage of this approach is that catcher probe DNA is stably bound to the nylon membrane principally by UV-light activated thymine bases interacting with the primary amine groups of the nylon matrix. This procedure permitted highly stringent assay conditions without a loss of the substrate molecule. Figure 20 shows the results of a hybridization between UV cross-linked SS DNA (clone KO1318-750) and a complementary labeled RNA probe (recombinant transcription plasmid KO64-750). Sensitivity levels were comparable to heat fixing DNA on nitrocellulose, thus a less fragile membrane solid support with equal or better sensitivity than nitrocellulose was provided.

To minimize background in this technique blots were treated post-hybridization with RNase A to remove detector probe which was nonspecifically bound to the nitrocellulose. Before and after treatment of blots with 100 ug/ml of RNase A are shown in Figure 21.

To determine the ability of the sandwich hybridization technique to detect dengue-infected mosquitoes, Aedes albopictus were intrathoracically inoculated with dengue-2 virus. Both radioactive and biotinylated detector probes were used in these assays. First attempts, using crude mosquito extracts failed to distinguish pools of infected mosquitoes from pools of uninfected mosquitoes (Figure 22). However, several plasmid or phage (KO65-1200 and KO1318-1200 constructs) used in the procedure were not

used in the previously reported sandwich hybridization protocol (see previous progress report). These were being recharacterized to assure that the probes are the correct polarity and size.

C. Publications/Manuscripts

The following manuscripts that detail much of the preceding information are in preparation for submission.

1. Chandler, L.J., Beaty, B.J., Bishop, D.H.L., and Ward, D.C. 1987. Detection of La Crosse and snowshoe hare viral nucleic acids by in situ hybridization. Submitted to Am J Trop Med Hyg.

2. Olson, K., Beaty, B.J., Blair, C.D., and Padmanabhan, R. 1987. Detection of Dengue RNA in cells and in mosquitoes by hybridization. J. Clin Micro, in preparation for submission.

VI. Discussion

Significant progress has been made in the areas of development and characterization of constructs and protocol development. Techniques for blotting and detecting LAC and DEN RNA species on nitrocellulose using both isotopic and biotinylated probes have been developed as well as techniques to detect LAC RNA in situ. Results thus far are most encouraging. However, two major problems or potential problems have been encountered which impede the use of hybridization in virus diagnosis and surveillance, especially in clinical or field situations. These are problems associated with sensitivity for detection of nucleic acid blotted onto nitrocellulose and the need for laborious, time consuming extraction procedures necessary to purify the RNA for processing in the current protocols.

For detection of dengue virus RNA immobilized on nitrocellulose, the sensitivity level is 1.5×10^6 genome equivalents. For LAC virus RNA even a greater concentration is required. Yet for LAC in situ hybridization, strong signal is detectable in cells when the titer is less than 3.0 log $TCID_{50}$ per ml. Thus, there seems to be an inherent problem involved in retaining RNA which has been blotted directly to nitrocellulose.

In our hands, phenol-chloroform extraction procedures have proven to be essential to obtain consistent, sensitive results. Such laborious and extensive procedures would be ill-suited to field or clinical situations.

Because of these difficulties or potential problems, sandwich hybridization techniques were developed to detect arbovirus NA in mosquito suspensions. These techniques appear to be more sensitive than conventional blot hybridization, presumably because RNA need not be immobilized on nitrocellulose.

The previously developed in situ hybridization procedure to detect LAC virus S RNA in cells was not specific. The probe used was a cDNA of the S RNA segment of LAC virus. Although the probe was sensitive for detection of analyte RNA, it was not specific. The S RNA sequences of SSH, TVT, TAH, JC, and CE all hybridized with the probe (Figure 2). Thus this probe construct

is group specific, detecting all tested members of the California group. This probe would be most useful in a screening test. In contrast, the SSH M RNA cDNA probe hybridized to SSH virus infected cells but not to LAC infected cells (Figure 3). Thus this probe is apparently type specific, permitting differentiation between LAC and SSH virus. Both group and type specific probes have their relative advantages in diagnosis. Application of hybridization techniques using such probes should provide group and type specific diagnosis of an etiologic agent in a clinically relevant time frame.

Studies were conducted to develop RNA probe systems for diagnosis. The RNA transcript probes were demonstrated to be specific (Figures 4 and 5), but the preliminary results do not suggest that sensitivity is signficantly greater than that attained with cDNA probes. This would suggest that the major determinant of sensitivity in the blot hybridization protocols that are used is the efficiency of RNA analyte binding to nitrocellulose (Table 3). Development of RNA probes will be continued because they are relatively easily prepared and used. In addition, strand specific probes can be prepared, adding a more sophisticated system for determination of viral replication mechanisms and dynamics.

Considerable effort was devoted to the development of catcher and detector probe systems. A variety of dengue specific SS DNA and RNA probes were constructed and have been used to detect dengue RNA by sandwich hybridization. So far nanogram quantities of purified RNA have been detected, but we are confident that the sensitivity of the technique can be undoubtedly be improved. Additional advantages include minimizing sample manipulation steps and precluding binding of analyte RNA directly onto the nitrocellulose. We are currently determining the efficacy of the technique for detection of dengue RNA in infected mosquitoes and mosquito pools.

V. Cost analysis of budget expenditures.

All budgetary expenditures have been made.

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Table 1. Comparison of the detection of La Crosse virus nucleic acid and antigen in a growth curve

Hrs PI	Antigen detection[a]			Hybridization[b]	
	HIAF	Anti-G1 814-02	Anti-N 807-18	^{35}S	Biotin
0	-	-	-	1+	-
2	1+	1+	-	-	-
4	4+	1+	1-2+	3+	0-1+
6	4+	2-3+	3+	4+	1+
8	4+	0-1+	4+	4+	2+
10	1-2+	0-1+	4+	4+	2-3+

[a]intensity of fluorescence
[b]intensity of signal

Table 2. Dengue probe constructs

Clone designation	Vector	Insert Data	Probe Type	Probe Sense
1. KOBR-1	pBR322	1.4 kb, pVV-1, PstI	DS-DNA	+/-
2. KOBR-17	pBR322	1.95kb, pVV-17, PstI	DS-DNA	+/-
3. KOBR-750	pBR322	750 bp, pVV-17, EcoRI-PstI	DS-DNA	+/-
4. KOBR-1200	pBR322	1200bp, pVV-17, EcoRI-PstI	DS-DNA	+/-
5. KOTZ-750	PTZr18	750 bp, pVV-17, EcoRI-PstI	SS-RNA	+
6. KOTZ-1200	PTZr18	1200bp, pVV-17, EcoRI-PstI	SS-RNA	-
7. KO64-750	pSP64	750 bp, pVV-17, EcoRI-PstI	SS-RNA	-
8. KO65-1200	pSP65	1200bp, pVV-17, EcoRI-PstI	SS-RNA	-
9. KO65-1	pSP65	1.4 kb, pVV-1, PstI	SS-RNA	+or-[a]
10. KO65-17	pSP65	1.95kb, pVV-17, PstI	SS-RNA	+or-
11. KO65-9	pSP65	1.6 kb, pVV-9, PstI	SS-RNA	+or-
12. KO1318-750	M13mp18	750 bp, pVV-17, EcoRI-PstI	SS-DNA	+
13. KO1319-750	M13mp19	750 bp, pVV-17, EcoRI-PstI	SS-DNA	-
14. KO1318-1200	M13mp18	1200bp, pVV-17, EcoRI-PstI	SS-DNA	-
15. KO1319-1200	M13mp19	1200 bp, pVV-17, Ecor1-Pst1	SS-DNA	+

[a]sense not yet determined

Table 3. RNA binding efficiencies to nitrocellulose

Sample[1]	CPM Initial	CPM Prehybridization	CPM Posthybridization	RNA (%)[2] Bound
1.	391,445	131,974	49,319	12.6
2.	186,225	72,208	20,567	11.0
3.	103,575	38,843	12,667	12.2
4.	46,590	19,065	6,206	13.3
5.	26,255	10,677	3,274	12.5
6.	12,735	5,024	2,369	18.6

[1] Counts given per sample is the mean of three replicate applications of the sample to nitrocellulose. Samples 2 through 6 represent sequential two-fold dilutions of sample 1.

[2] % RNA bound was determined by the formula:

$$\frac{\text{CPM posthybridization}}{\text{CPM initial}} \times 100$$

Table 4. Results of virus isolation from mosquito pools.

Pool	No. of uninfected mosquitoes	No. of infected mosquitoes	Total No. of mosquitoes	Log_{10} TCID50 titer
A	0	25	25	5.25
B	13	12	25	4.75
C	20	5	25	3.25
D	24	1	25	2.75
E	25	0	25	0.00

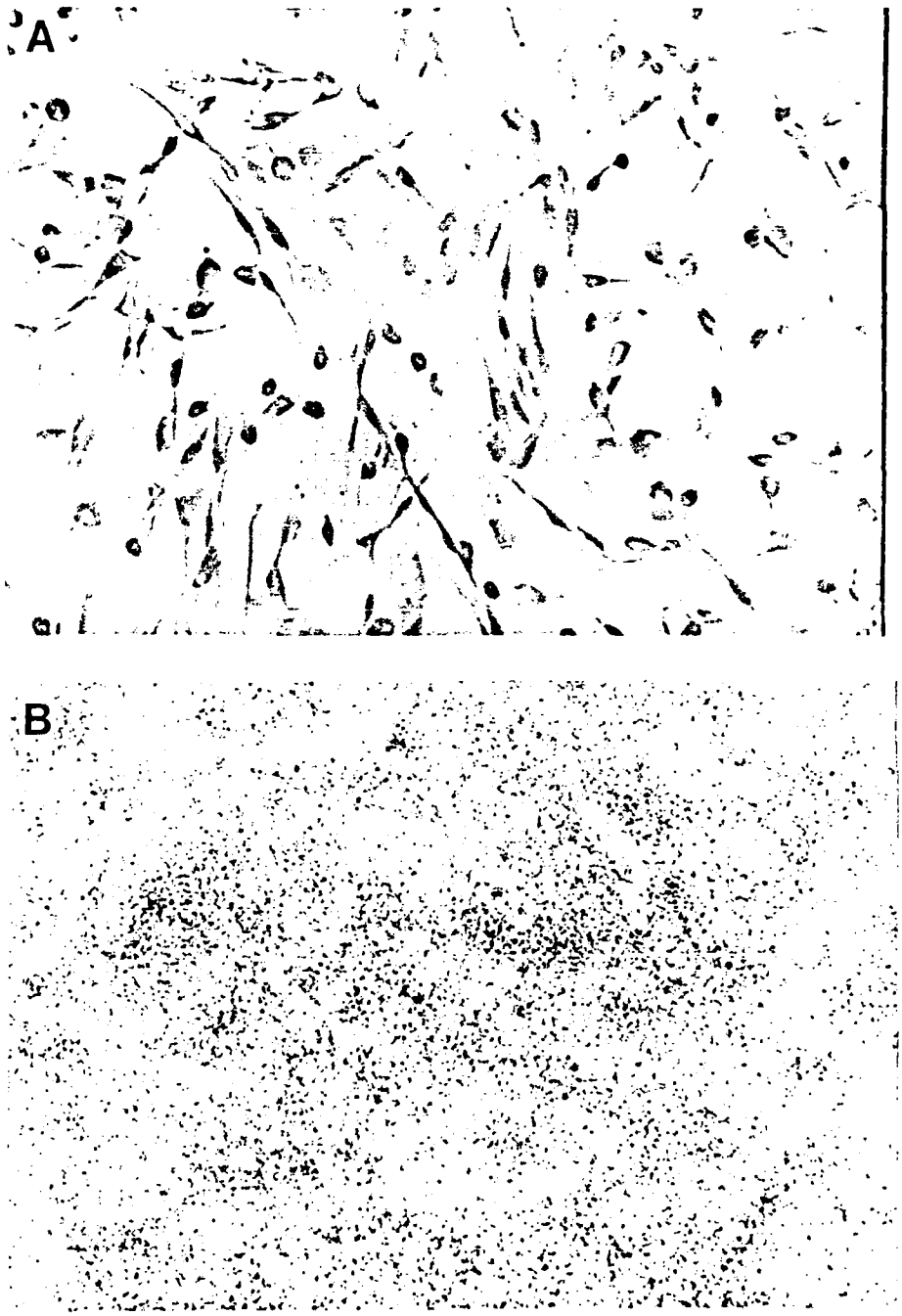


Figure 1. BHK-21 cells hybridized with ^{35}S labeled LAC S RNA cDNA probe
A. Uninfected cells.
B. LAC infected cells.

Figure 2

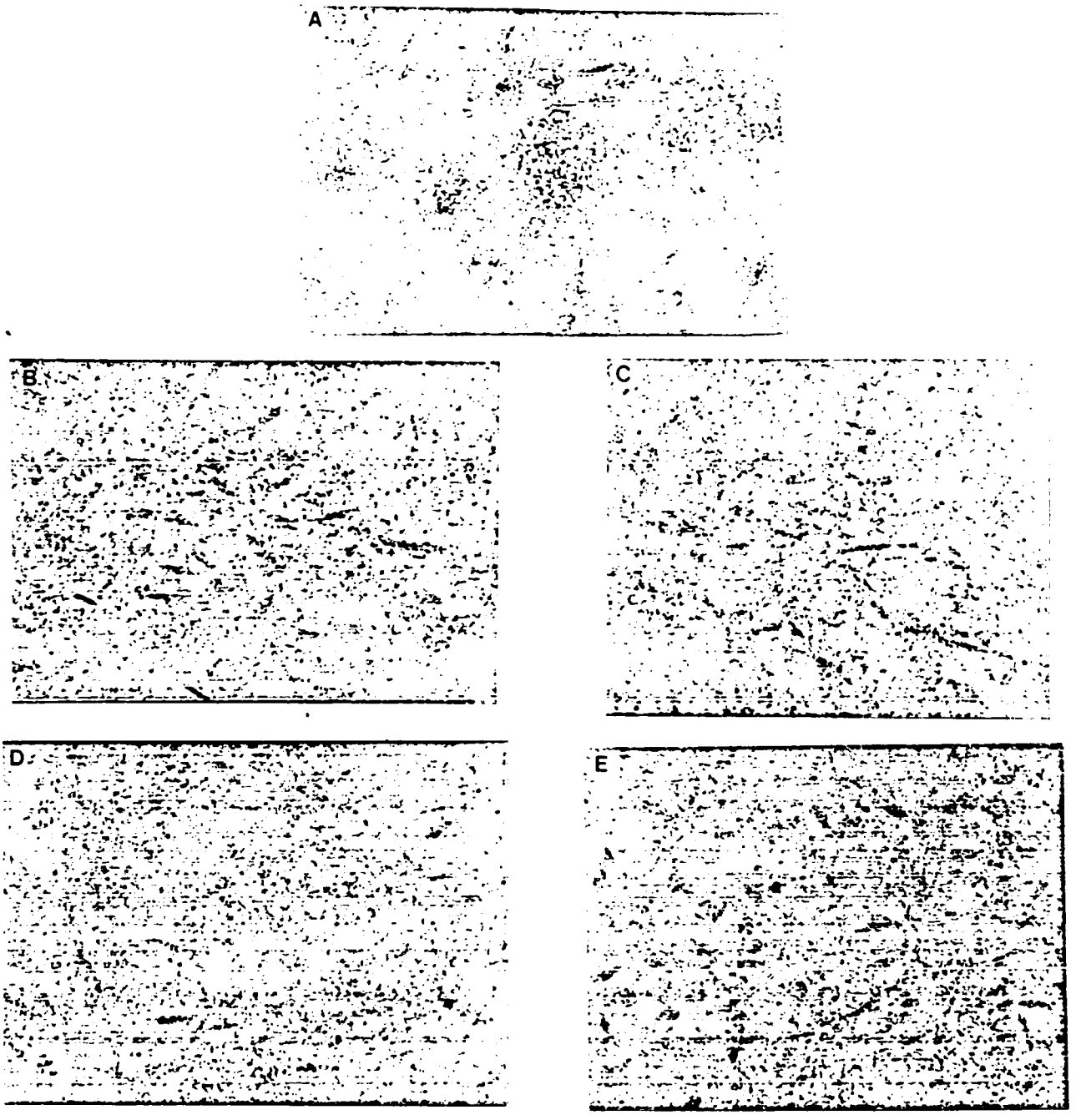


Figure 2. Specificity of ^{35}S labeled LAC S RNA cDNA probe.
A. SSH infected cells. B. CE infected cells.
C. TVT infected cells. D. TAH infected cells.
E. JC infected cells.

Figure 2

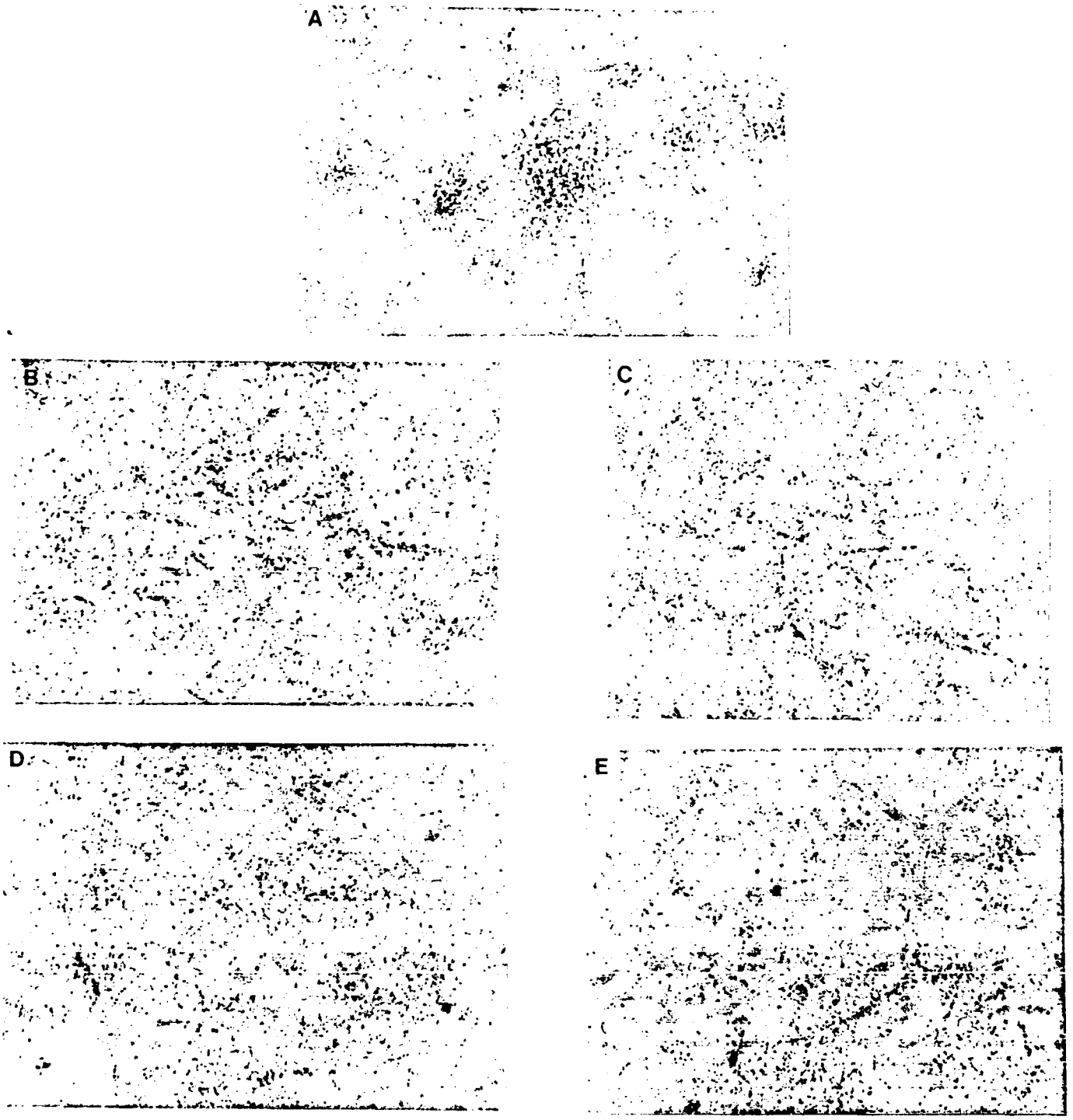


Figure 2. Specificity of ^{35}S labeled LAC S RNA cDNA probe.
A. SSH infected cells. B. CE infected cells.
C. TVT infected cells. D. TAH infected cells.
E. JC infected cells.

Figure 3.

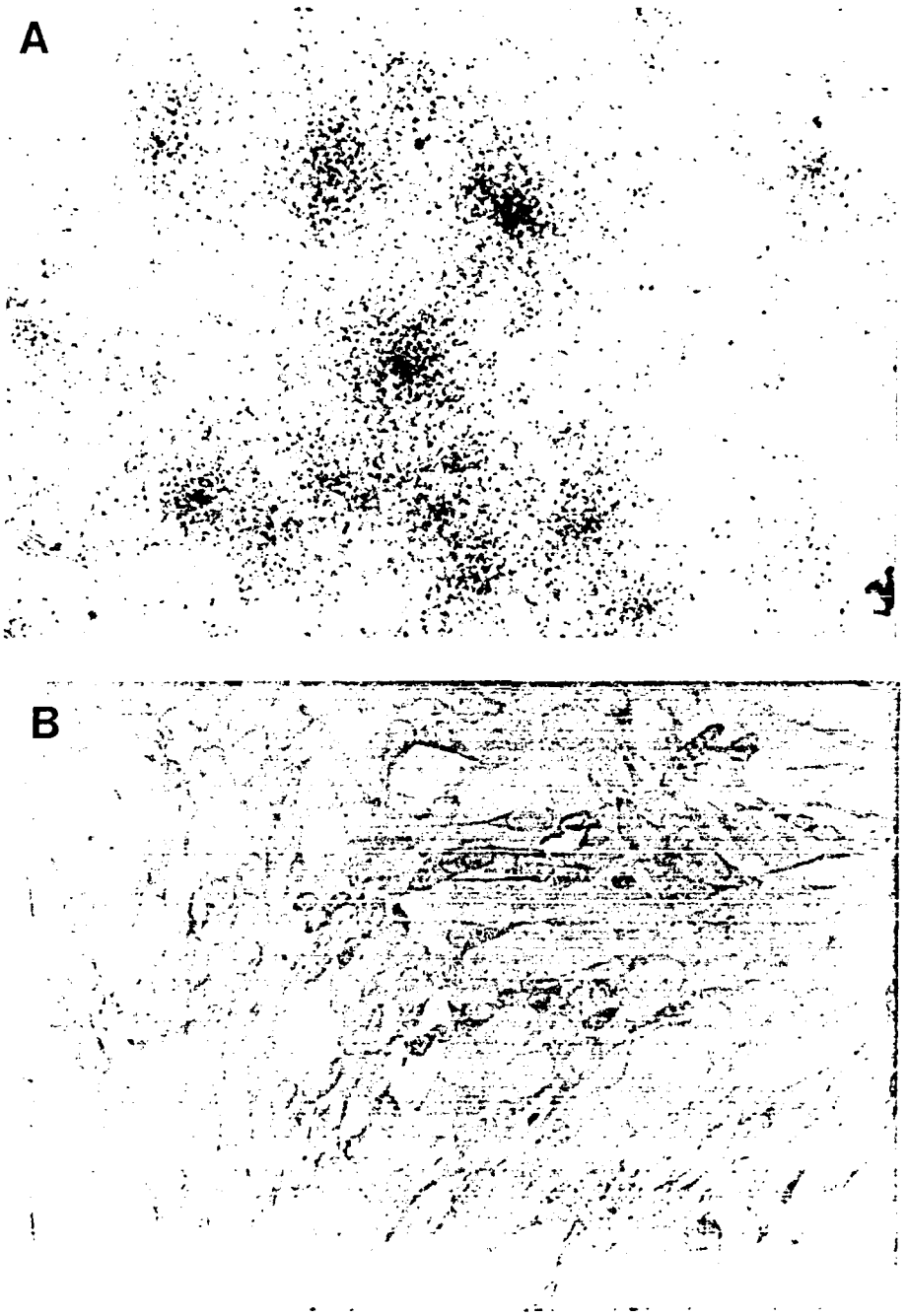


Figure 3. Specificity of ^{35}S labeled SSH M RNA cDNA. Cells hybridized with ^{35}S labeled SSH M60-78 cDNA probe.
A. SSH infected cells. B. LAC infected cells.

Figure 4. Detection of La Crosse virus nucleic acid in cell culture suspensions using an ^{35}S labeled RNA probe.

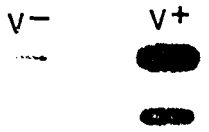


Figure 5. Detection of La Crosse virus nucleic acid in cell culture suspensions using a ^{32}P labeled RNA probe.

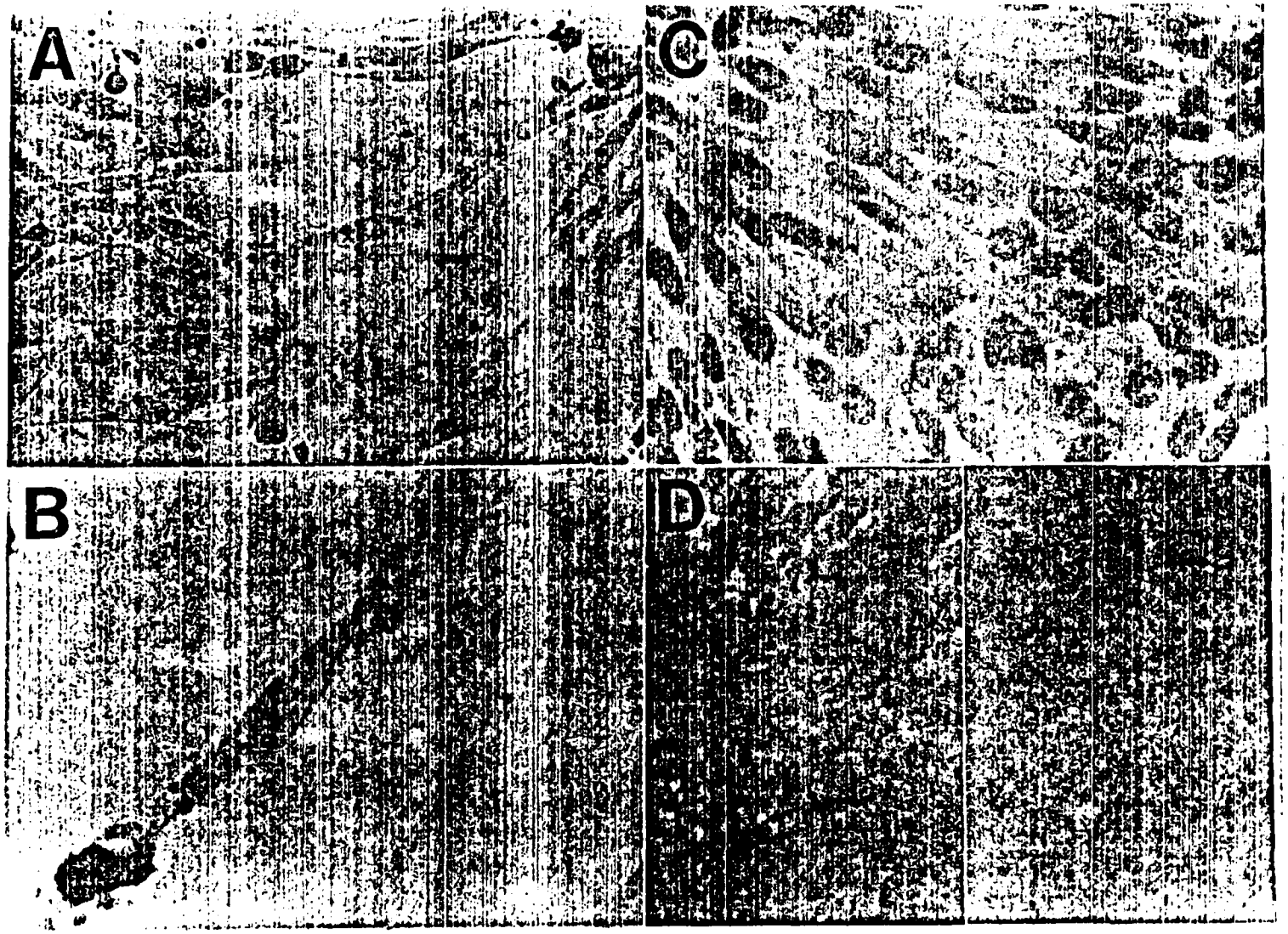
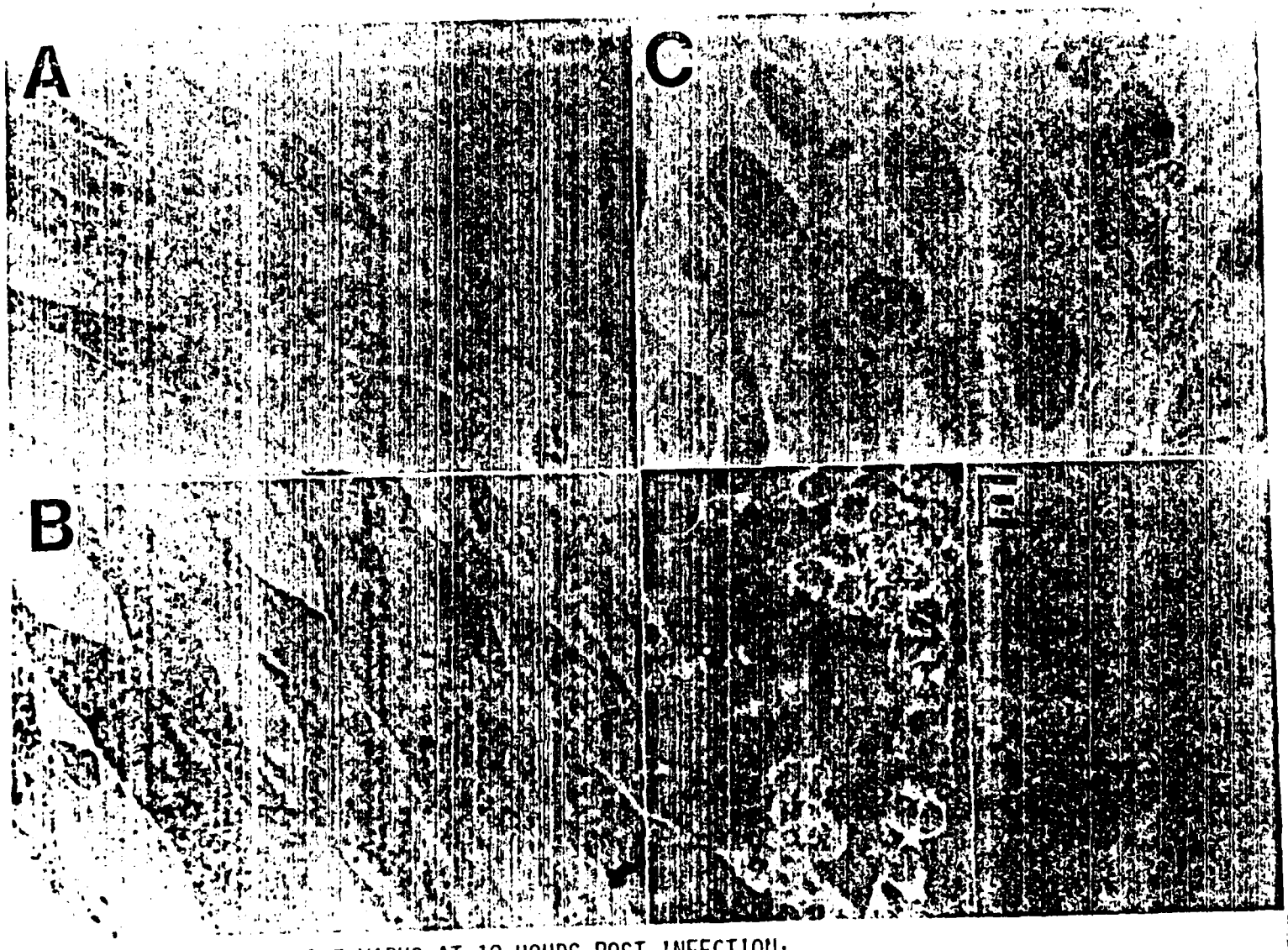


Figure 6

DETECTION OF LACROSSE VIRUS AT 6 HOURS POST INFECTION:

A. VIRUS S RNA BY IN SITU HYBRIDIZATION (400X)
B. VIRUS S RNA BY IN SITU HYBRIDIZATION (1000X, NOMARSKI OPTICS)
C. UNINFECTED CELLS - HYBRIDIZATION CONTROL (400X, NOMARSKI OPTICS)
D. VIRUS ANTIGEN BY IMMUNOFLUORESCENCE (400X)
E. UNINFECTED CELLS - IMMUNOFLUORESCENCE CONTROL (1000X)



DETECTION OF LACROSSE VIRUS AT 12 HOURS POST INFECTION:

A. VIRUS S RNA BY IN SITU HYBRIDIZATION (1000X)
B. VIRUS S RNA BY IN SITU HYBRIDIZATION (1000X, NOMARSKI OPTICS)
C. UNINFECTED CELLS - HYBRIDIZATION CONTROL (1000X, NOMARSKI OPTICS)
D. VIRUS ANTIGEN BY IMMUNOFLUORESCENCE (400X)
E. UNINFECTED CELLS - IMMUNOFLUORESCENCE CONTROL (1000X)

Figure 8

DETECTION OF LACROSSE VIRUS AT 24 HOURS POST INFECTION:

A. VIRUS S RNA BY IN SITU HYBRIDIZATION (1000X)
B. VIRUS S RNA BY IN SITU HYBRIDIZATION (1000X, NOMARSKI OPTICS)
C. UNINFECTED CELLS - HYBRIDIZATION CONTROL (1000X, NOMARSKI OPTICS)
D. VIRUS ANTIGEN BY IMMUNOFLUORESCENCE (1000X)
E. UNINFECTED CELLS - IMMUNOFLUORESCENCE CONTROL (1000X)

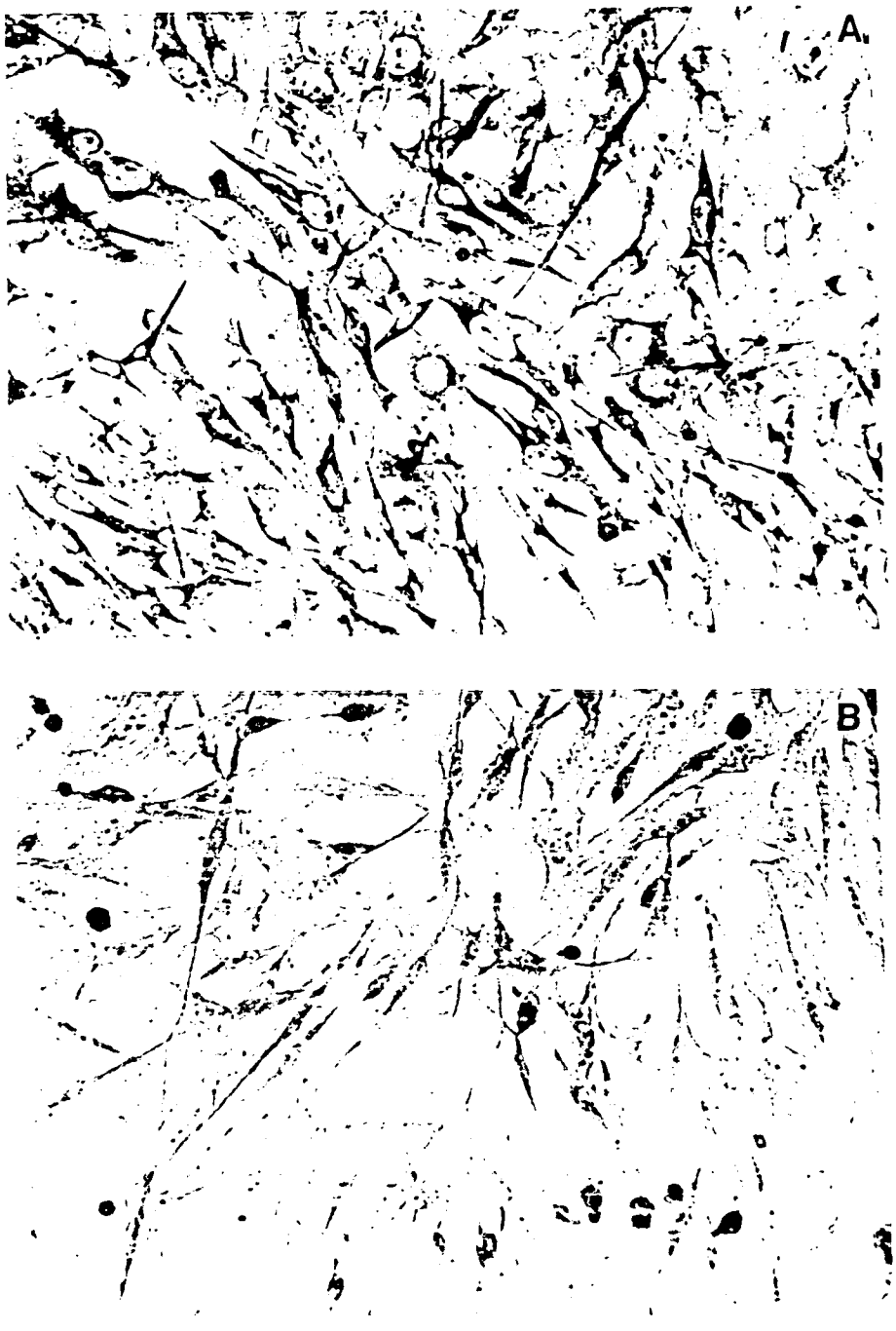


Figure 9. Detection of LAC virus RNA in BHK-21 cells. Biotin-labeled pLACs17, direct ABC-AP detection system. A. LAC infected cells. B. Uninfected cells.

Figure 10. Detection of La Crosse virus nucleic acid in cell culture suspensions using a biotinylated cDNA probe.

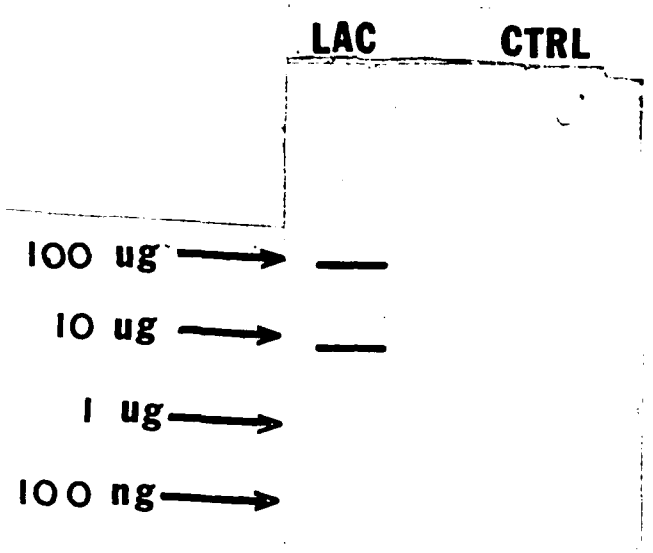
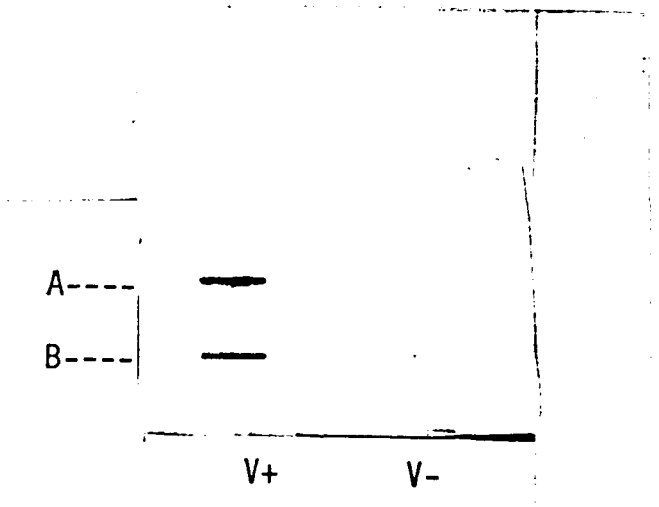
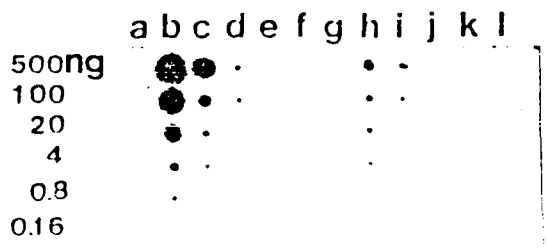
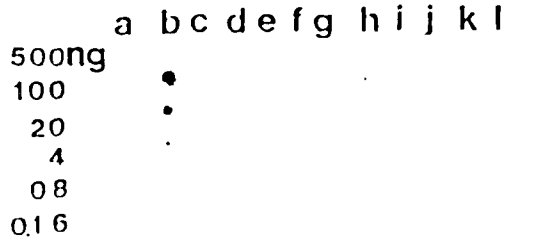


Figure 11. Detection of La Crosse virus nucleic acid in mosquito suspensions using a biotinylated cDNA probe.

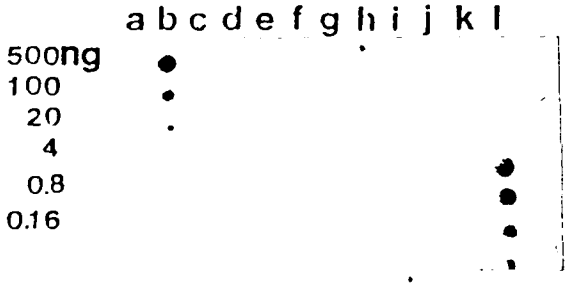




pVV1 probe



pVV9 probe



pVV17 and pUC-13-1 probe

pUC-13-1 probe

Figure 12. Sequence specificities of dengue-2 cDNA. Recombinant plasmids pVV1, pVV9, and pVV17, as well as plasmid pUC-13-1 were nick translated using ^{32}P-dCTP and hybridized to dot blots containing 5-fold dilutions of the following RNAs: A) dengue-1, B) dengue-2, C) dengue-3, D) dengue-4, E) LAC, F) West Nile, G) dengue-1, H) dengue-2, I) dengue-3, J) dengue-4, K) uninfected mosquito cell lysates. RNAs from rows A-F were extracted from purified virus and RNAs from rows G-J were extracted from mosquito cell lysates. In row L, the first 4 spots contain no RNA; the last 4 spots contain pUC-8 DNA which contains sequences in common with pUC-13-1.

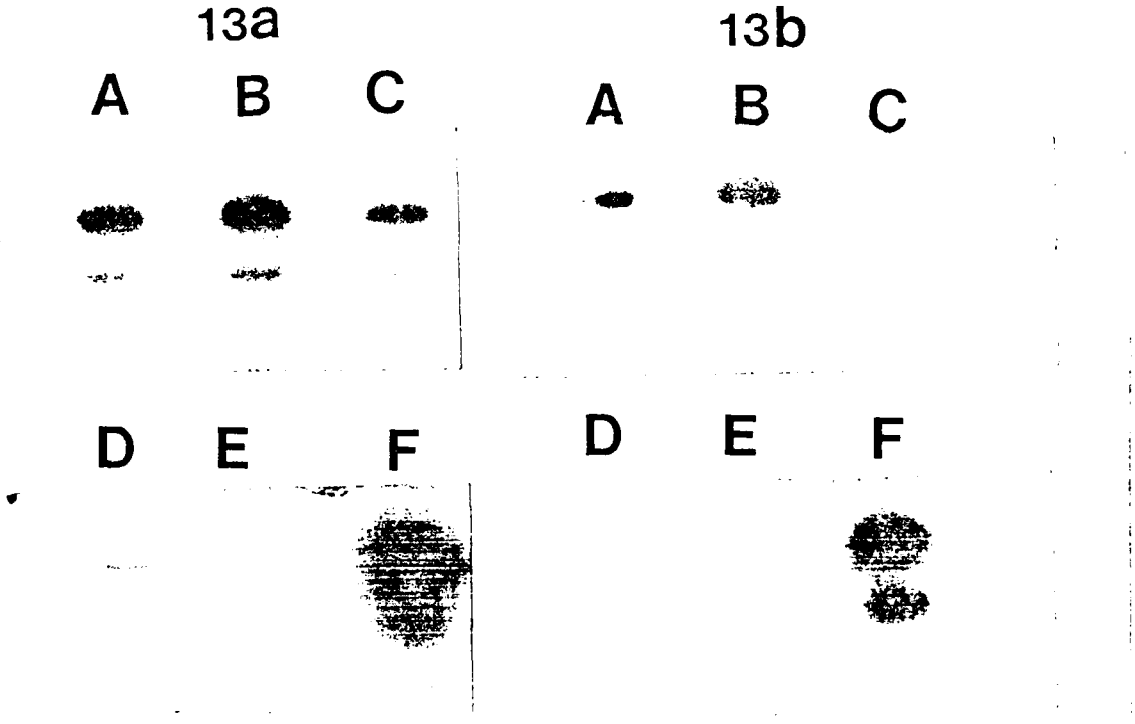


Figure 13. Direct detection of dengue-2 RNA from infected Aedes albopictus. Mosquito RNA was extracted from pool consisting of 25 infected mosquitoes (A); 12 infected, 13 uninfected mosquitoes (B); 5 infected, 20 uninfected mosquitoes (C); 1 infected, 24 uninfected mosquitoes (D); and 25 uninfected mosquitoes (E). Sample F is dengue-2 RNA purified from virus. Each sample was serially diluted tenfold before application. Dengue-2 RNAs in samples A through F were detected by hybridization using transcription plasmids KO65-750 (13a) and KO65-1200 (13b).

Figure 14a. pVV recombinant plasmid constructs containing the dengue-2 cDNAs of 1.95kb, 1.65kb, and 1.4kb. A unique EcoRI site has been identified in the 1.95kb cDNA and was utilized in construction of the probes for the sandwich hybridization.

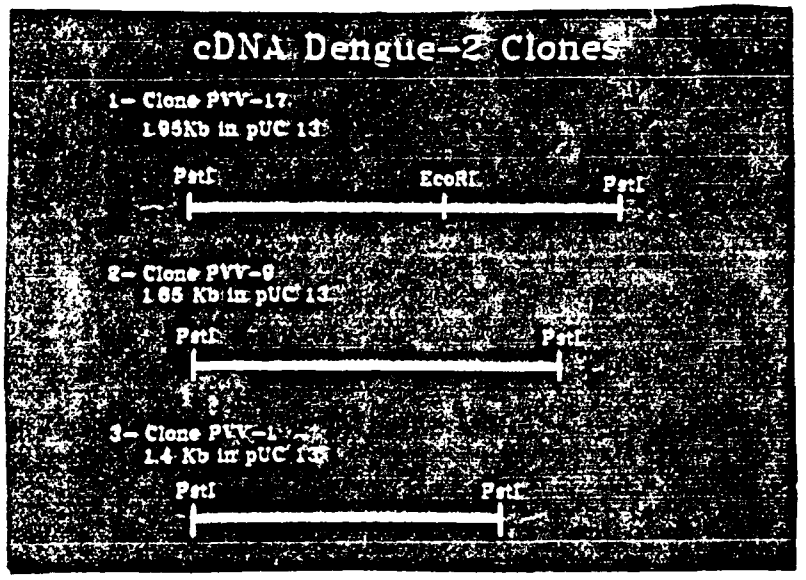
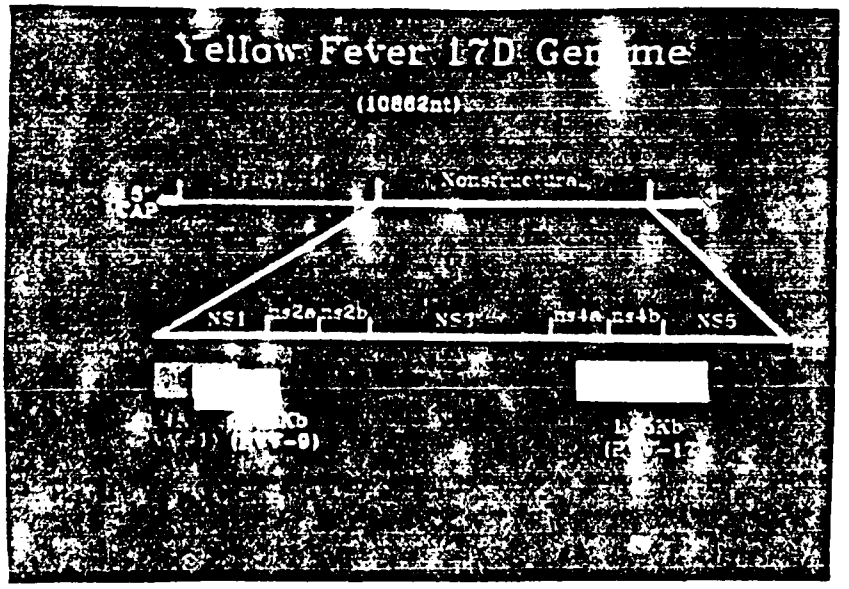


Figure 14b. Relative locations of the dengue cDNAs on the yellow fever genome (by base sequence homology).



Detector Probe Constructions

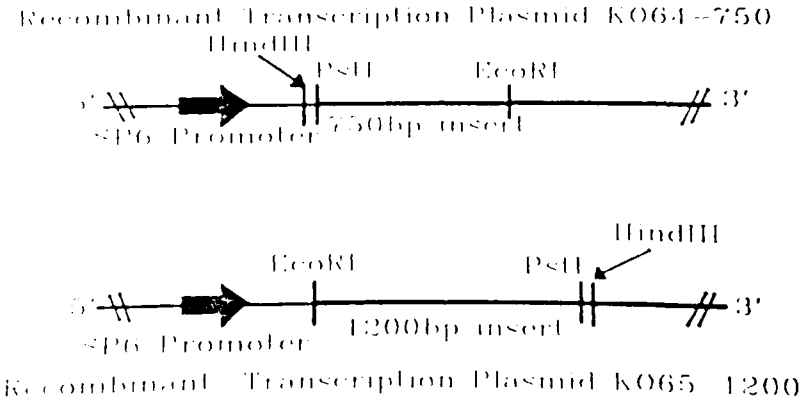


Figure 15. Detector probe constructs. K064-750 and K065-1200 detector probes were produced by inserting the 750bp and 1200bp cDNAs into pSP-64 and pSP-65 RNA transcription plasmids. Inserted DNAs were isolated from PstI-EcoRI digests of construct pV717. Both constructs generate (-) sense RNA complementary to the dengue genomic RNA.

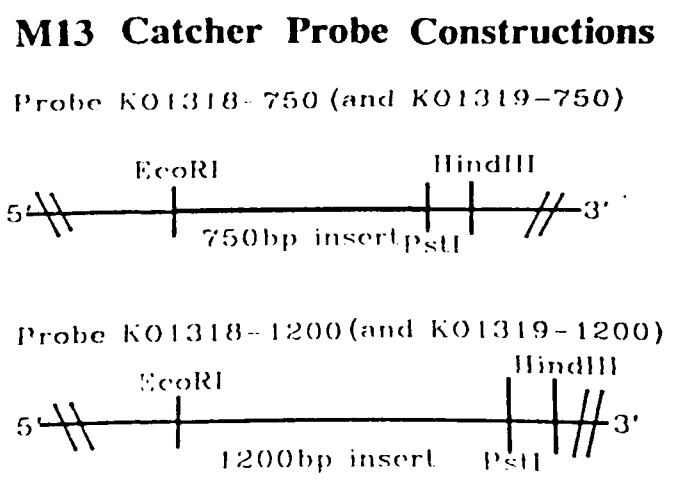


Figure 16. Catcher probe constructs. KO1319-750 and KO1318-1200 catcher probes were produced similarly to that described above (Figure 15). These constructs generated single-stranded DNAs complementary to dengue genomic RNA.

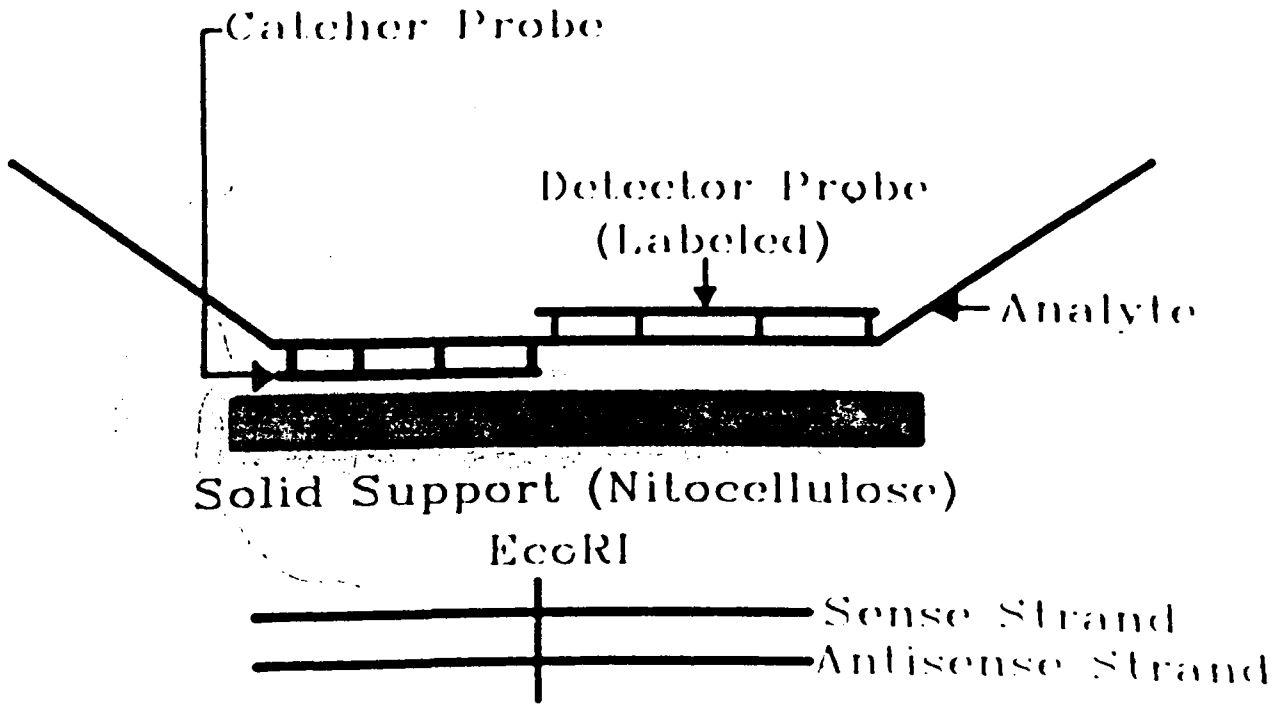


Figure 17. Sandwich hybridization schematic.

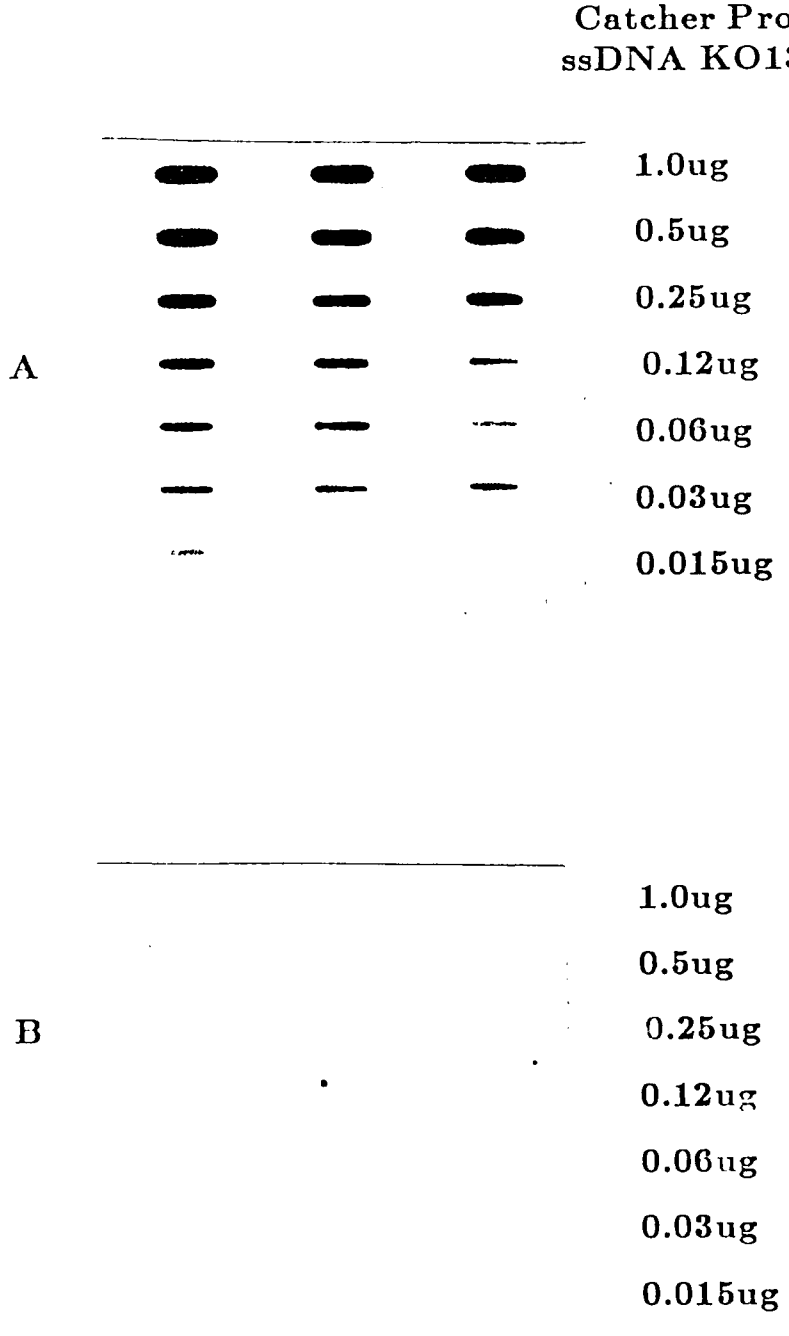


Figure 18. Detection of dengue virus RNA by sandwich hybridization. A) Purified dengue-2 RNA was the analyte; 100 ng of viral RNA was used in hybridization; 0.25 to 0.5 ug of the catcher probe is adequate to detect viral RNA. B) No viral RNA was added.

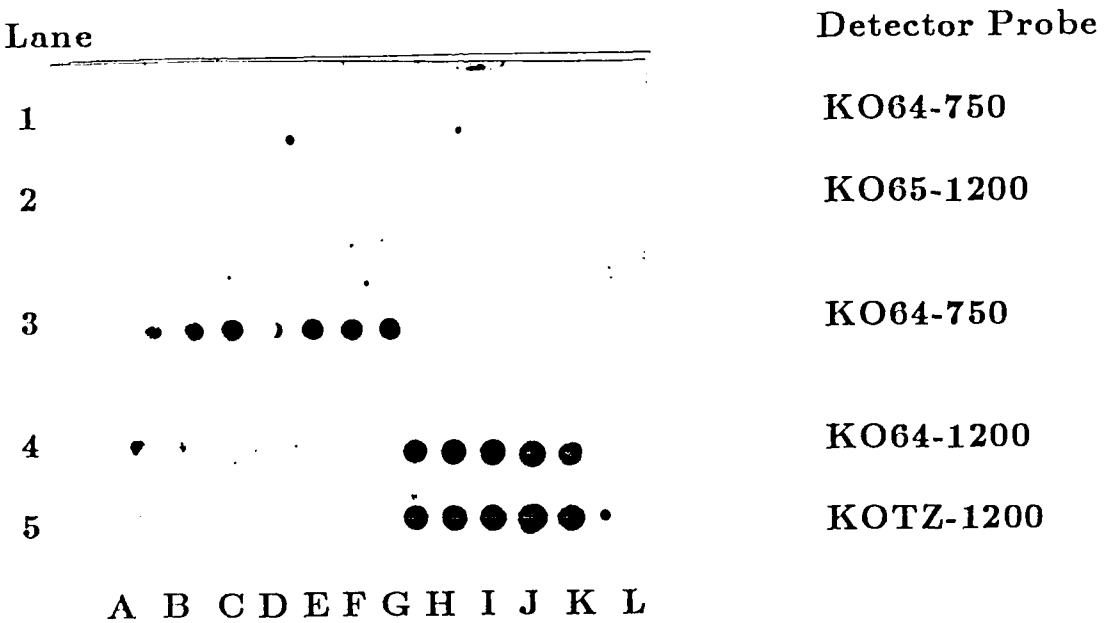


Figure 19. Sandwich hybridization using different detector and catcher probes.Analyte for lanes 1 and 2 was a (-) strand RNA transcribed from construct KO65-1950A. For lanes 3,4, and 5 the analytes were (+) strand transcribed from the construct KO65-1950B. The catcher probes were as follows: rows A and B M13mp18 RF; rows C-G KO1318-1200 single-stranded DNA; rows H-L KO1319-750 single-stranded DNA. All capture probes were applied at 1.0 ug/spot. 100ng of analyte was used to hybridize each of 5 test strips.

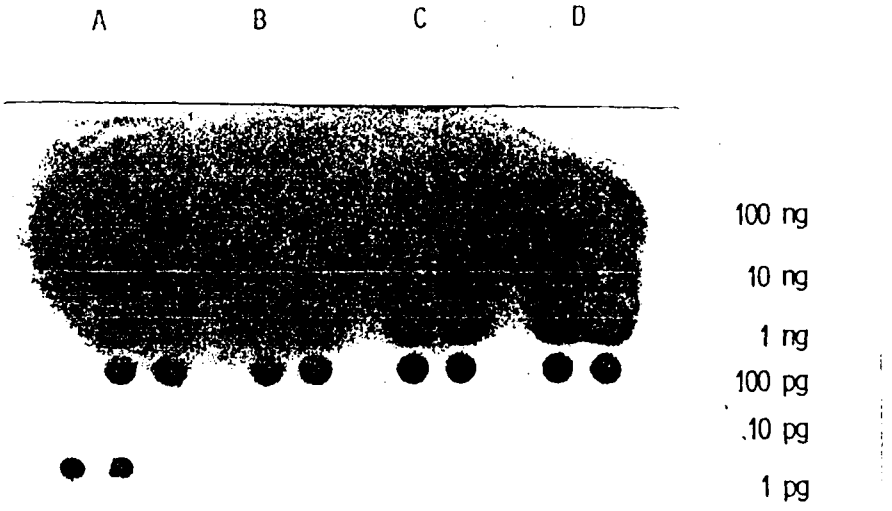


Figure 20. Results of ultraviolet light cross-linking of DNA to a nylon filter (Nytran). Samples A-D contain identical DNAs cross-linked to the filter with each group composed of a negative control (single-stranded M13mp18 DNA) followed by duplicate positive sense single-stranded DNAs generated from recombinant phage KO1318-750. DNAs of group A-D are cross-linked by exposure to a UV source for 3 min, 4 min, 5 min, and 6 min respectively (160 kJ/m^2). The probe is a ^{32}P-CTP labeled transcript from clone KO64-750 and complementary to the KO1318-750 insert cDNA.

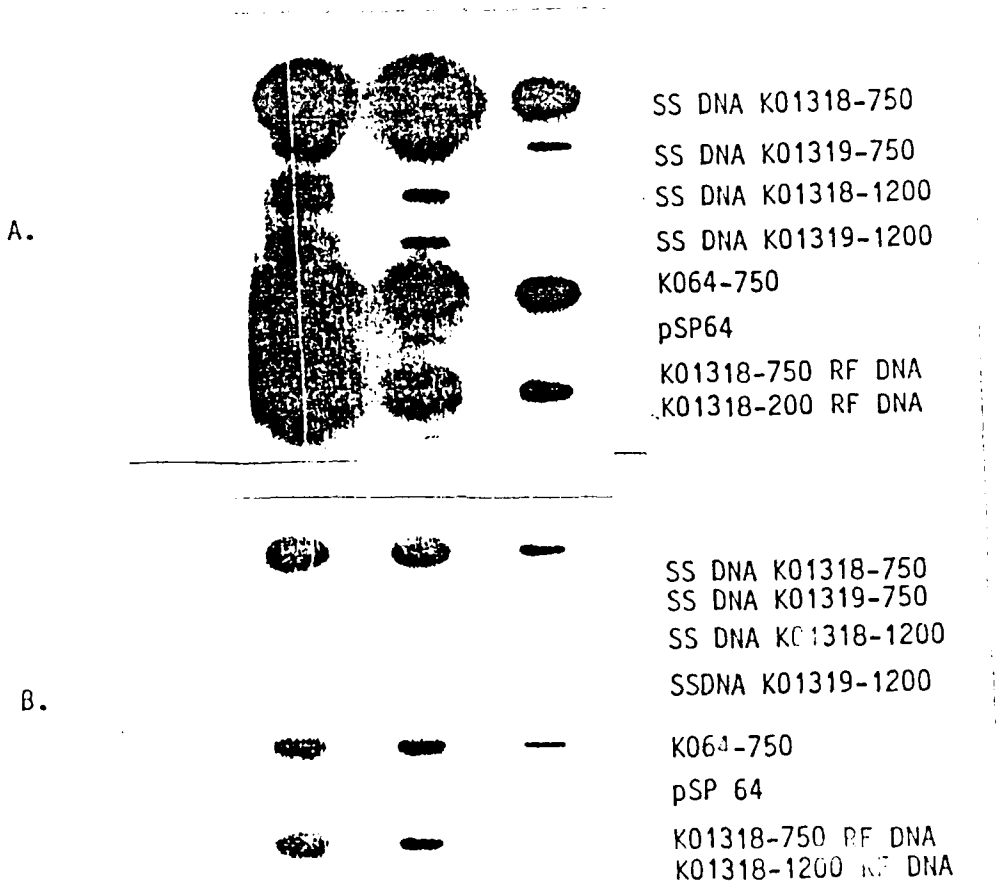


Figure 21. Effect of RNase A treatment of blots to reduce background. A) blots untreated and B) blots treated with 100 ug/ml RNase A for 15 min at room temperature. Probe used in hybridizations of blots A and B was an RNA probe transcribed from clone K064-750.

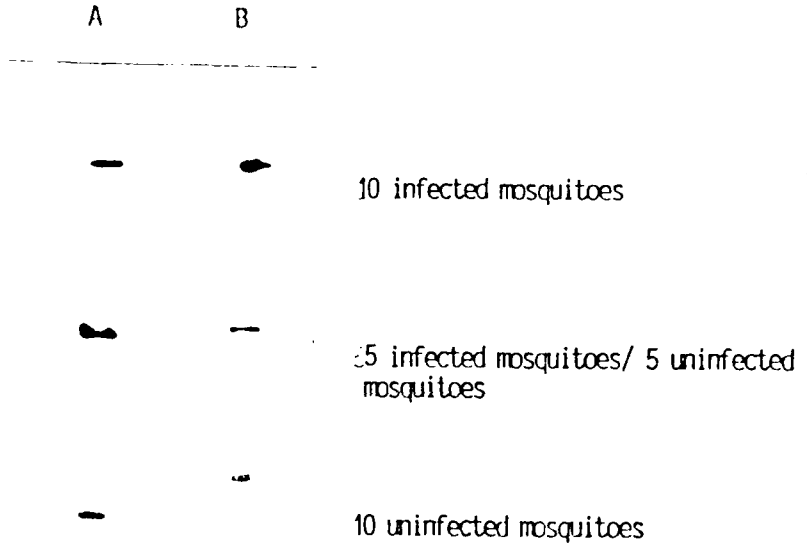


Figure 22. Results of analyzing mosquito pools for the presence of dengue-2 RNA sequences by the sandwich hybridization technique. Aedes albopictus were inoculated intrathoracically with virus and RNA was extracted 14 days later. A) Sandwich hybridization using KO1318-1200 single-stranded DNA catcher probe and labeled KO64-750 RNA as detector probe. B) Sandwich hybridization using KOBR-1200 as catcher probe and labeled KO64-750 RNA as detector probe.

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